

Interferon- γ acts directly on CD8⁺ T cells to increase their abundance during virus infection

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Interferon- γ (IFN γ) is important in regulating the adaptive immune response, and most current evidence suggests that it exerts a negative (proapoptotic) effect on CD8⁺ T cell responses. We have developed a novel technique of dual adoptive transfer, which allowed us to precisely compare, in normal mice, the in vivo antiviral responses of two T cell populations that differ only in their expression of the IFN γ receptor. We use this technique to show that, contrary to expectations, IFN γ strongly stimulates the development of CD8⁺ T cell responses during an acute viral infection. The stimulatory effect is abrogated in T cells lacking the IFN γ receptor, indicating that the cytokine acts directly upon CD8⁺ T cells to increase their abundance during acute viral infection.

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IFN γ is produced by effector CD8⁺ T cells, Th1 CD4⁺ T cells, NK, and NK T cells. Its receptor, which is expressed on many cell types, is a heterodimer of IFN γ R1 and IFN γ R2, both of which are required for IFN γ signaling (1). IFN γ is essential for the control of many microbial infections, acting very early to reduce the infectious burden; mutations in either the cytokine or its receptor lead to recurrent bacterial or parasitic infections in humans (2). The important role of IFN γ in immune defense is further highlighted by the fact that several viruses encode proteins designed to interfere with IFN γ R signaling (3, 4). IFN γ also modulates the adaptive immune response. It has several indirect effects on CD8⁺ T cell activity: for example, IFN γ induces expression of the immunoproteasome, the TAP transporter proteins and MHC class I molecules, thus rendering intracellular pathogens more visible to the CD8 wing of the adaptive immune response. These indirect effects of IFN γ should generally enhance CD8⁺ T cell activity but, conversely, its direct effects on T cells are thought to be negative (suppressive). T cells treated with IFN γ show reduced proliferation and/or increased apoptosis (5, 6), and mice lacking IFN γ generate greater numbers of *Listeria*-specific CD8⁺ T cells than do wild-type mice (7); IFN γ also is thought to eliminate *Mycobacterium*-specific CD4 T cells by inducing apoptosis (8). IFN γ favors Th1 cell differentiation of CD4 T cells, and inhibits Th2 cell differentiation, apparently through down-regulation of the IFN γ R on Th1 cells, which protects them from the suppressive effects

of IFN γ ; whereas Th2 cells continue to express the receptor, and are suppressed by the cytokine (9, 10). Therefore, current understanding holds that the direct effects of IFN γ on T cells are suppressive, and possibly proapoptotic.

Our laboratory has recently reported that IFN γ produced by CD8⁺ T cells plays a key role in establishing the differences in abundance between dominant and subdominant CD8⁺ T cell populations (i.e., IFN γ is required for immunodominance; 11). Furthermore, we have found that the rapidity of IFN γ expression by CD8⁺ T cells correlates with their ultimate abundance; fast-expressing cells become more numerous than slow expressers (12). These data could be explained by either positive or negative effects of IFN γ (i.e., IFN γ could regulate the relative abundances of different T cell populations either by actively stimulating some cell populations, or by actively suppressing others); and these regulatory effects on T cell abundance could be indirect (for example, via antigen-presenting cells) or direct. The present study was designed to determine (a) whether the effect of IFN γ on CD8⁺ T cells was stimulatory or suppressive; and (b) whether the cytokine acted directly on T cells, or indirectly.

RESULTS AND DISCUSSION

CD8⁺ T cell abundance is markedly reduced in the absence of IFN γ or IFN γ R

The primary CD8⁺ T cell responses to acute LCMV infection were compared in mice genetically deficient in either IFN γ ($\gamma^{-/-}$) or IFN γ R1 (γ R^{-/-}). After infection, the mice displayed

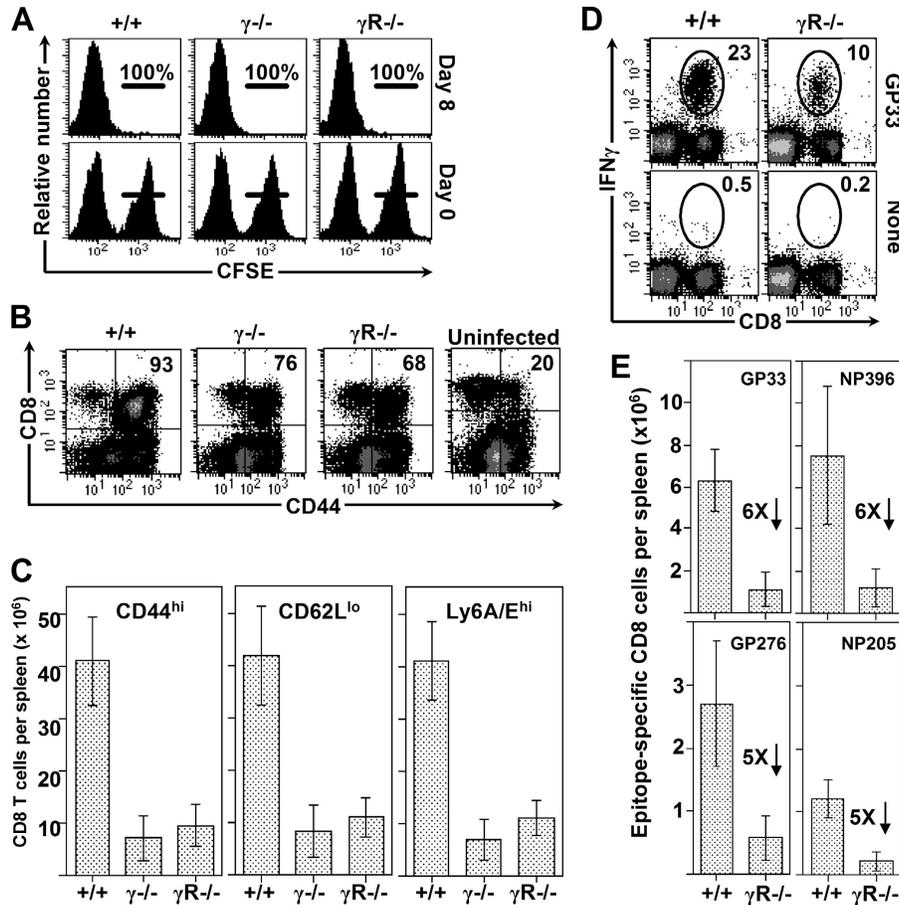


Figure 1. CD8⁺ T cell abundance is markedly reduced in the absence of IFN γ or IFN γ R. (A) The CTL response induced in IFN γ ^{-/-}, IFN γ R^{-/-}, and control C57BL/6 mice 8 d after infection was measured in vivo by injecting GP33-coated CFSE^{hi} and uncoated CFSE^{lo} target cells into mice. The numbers indicate the percentage of peptide-coated cells deleted in the recipient mice. (B) Dot plots show CD44 expression by splenocytes at 8 d after infection. An uninfected C57BL/6 mouse is shown for comparison. (C) Bar graphs depict the number of activated cells that are CD44^{hi}, CD62L^{lo}, or Ly6A/E^{hi} in infected mice. (D) The virus-specific responses in wild-type

and IFN γ R^{-/-} mice were quantified at day 8 after infection by ICCS. GP₃₃₋₄₁-specific cells are identified by ovals, and the numbers indicate the percentage of specific cells among all CD8⁺ T cells. (E) The numbers of epitope-specific CD8⁺ T cells, based on the spleen cell count and intracellular staining, are shown, along with the fold reductions between +/+ and γ R^{-/-} mice. The bars represent the average \pm SD from five or six mice per group, from four independent experiments. For each group, the p-value compared with +/+ mice is <0.001 (unpaired Student's *t* test).

strong in vivo virus-specific cytotoxicity (Fig. 1 A), consistent with earlier in vitro reports (13, 14), and they retained the capacity to reduce viral burden by several orders of magnitude (unpublished data). However, as judged by CD44 expression, lower proportions of CD8⁺ T cells were activated in γ ^{-/-} and γ R^{-/-} mice (Fig. 1 B), and this reduction was magnified by an ~3-fold reduced cellularity in the spleens; as a result, mice deficient in the IFN γ pathway had 5- to 10-fold fewer activated T cells (Fig. 1 C). This 80–90% reduction occurs in the virus-specific population, as revealed by ICCS analyses in wild-type and IFN γ R^{-/-} mice: there was a two- to threefold lower frequency of responding (IFN γ ⁺) cells in the receptor-deficient mice (Fig. 1 D) which, together with the reduced cellularity, resulted in a profound reduction in antigen-specific T cell numbers (Fig. 1 E). These data indicate that (contrary to current dogma) IFN γ strongly stimulates CD8⁺ T cell responses.

Expression of the IFN γ R1 on CD8⁺ T cells varies over the course of viral infection

Next, the expression of IFN γ R1 on CD8⁺ T cells was evaluated over the course of virus infection (Fig. 2 A). 14% of CD8⁺ T cells expressed the receptor before infection and, 8 d after infection, 83% were γ R1⁺. IFN γ R1 expression also was up-regulated on CD8⁺ T cells after infection of IFN γ ^{-/-} mice (Fig. 2 B), consistent with IFN γ -independent, TCR-mediated, induction of the receptor, as suggested previously (15). The kinetic analysis of γ R1 expression (Fig. 2 A) indicated that there was a brief decrease in the proportion of CD8⁺ T cells expressing γ R1 at 1 d after infection. The mechanism underlying this event is unknown, but at this early time after infection, innate immune responses are predominant (16); however, a role for adaptive immunity cannot be excluded, because recent data have shown that naive T cells

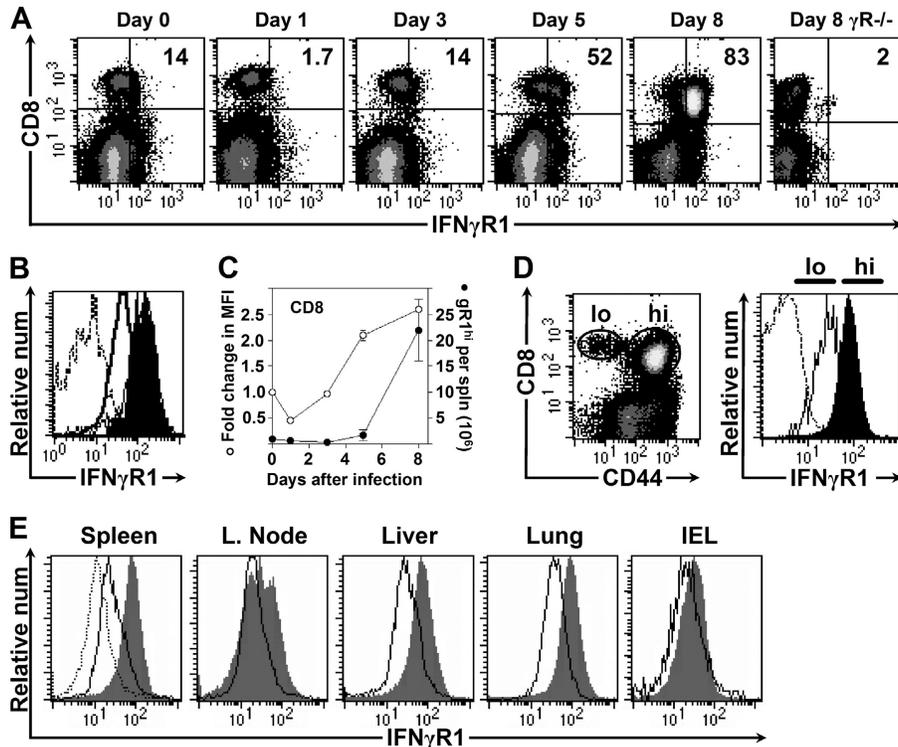


Figure 2. Expression of the IFN γ R on CD8 $^{+}$ T cells varies over the course of viral infection. (A) Representative dot plots show changes in expression of IFN γ R1 on T cells after infection. The percentages of CD8 $^{+}$ T cells expressing elevated amounts of the receptor are shown. (B) IFN γ R $^{-/-}$ CD8 $^{+}$ T cells express normal amounts of IFN γ R1 before infection (unshaded histogram), and up-regulate expression 8 d after infection (shaded histogram). Dotted line shows an isotype control antibody. (C) Changes over time in the geometric mean fluorescence of IFN γ R1 on wild-type splenic

CD8 $^{+}$ T cells (○) and the number of IFN γ R1 $^{+}$ cells per spleen (●). (D) Resting (CD44 lo) and activated (CD44 hi) CD8 $^{+}$ T cells are identified by a dot plot (left) and expression of IFN γ R1 among these cells is shown by the histograms (right). Dotted line shows an isotype control antibody. (E) IFN γ R1 expression on CD8 $^{+}$ T cells in the indicated tissues of uninfected mice (unshaded) or of day 8 mice (shaded). The dotted histogram shows the decreased expression in spleen at one day after infection; in the other tissues, the change in fluorescence at this time was minimal.

elaborate IFN γ within hours of first encountering cognate antigen (17). This transient down-regulation of γ R1 is followed by progressively increased expression from days 3 to 8 of infection; and, as shown in Fig. 2 C, both the number of CD8 $^{+}$ T cells expressing the receptor (●), and their level of expression (MFI; ○), increase as the infection proceeds. Elevated γ R1 expression was associated with activated, CD44 hi cells (Fig. 2 D). Similar changes in γ R1 expression were seen in other peripheral tissues (Fig. 2 E), indicating that the changes observed in the spleen probably represent cell-intrinsic alterations of expression, rather than changes in migratory patterns (for example, γ R1 $^{+}$ cells entering and γ R1 $^{-}$ cells exiting the spleen).

IFN γ exerts its effect directly on CD8 $^{+}$ T cells

The observation that IFN γ R levels on CD8 $^{+}$ T cells change over the course of infection (Fig. 2) is consistent with the hypothesis that IFN γ acts directly on CD8 $^{+}$ T cells to modulate their abundance. The diminished abundance of CD8 $^{+}$ T cells in IFN γ R $^{-/-}$ mice (Fig. 1) also is consistent with this hypothesis; however, since none of the cells in these knockout mice express the receptor, it remains possible that the effect of IFN γ on T cells is indirect. Furthermore, the interpretation of

T cell data after infection of IFN γ or IFN γ R mice is difficult, because these mice show altered clearance of virus, and the increased viral burden and/or antigen load might alter the T cell response. To determine whether the effects of IFN γ on CD8 $^{+}$ T cells are direct, and to circumvent the issues relating to virus/antigen load, we have developed a novel adoptive transfer model. Spleen cells from wild-type mice congenic for the Ly5a marker (Thy1.2, Ly5a) were mixed with spleen cells from γ R $^{-/-}$ (Thy1.2, Ly5b) mice, so that the number of CD8 $^{+}$ T cells in each group was equal. The cells were transferred into unirradiated, wild-type mice (Thy1.1, Ly5b) that were infected 1–2 d later. These recipient mice are fully immunocompetent, and eradicate the virus infection with normal kinetics. 8 d after infection, the responding CD8 $^{+}$ donor cells (all Thy1.2) can be readily identified (Fig. 3 A). Among these donor CD8 $^{+}$ T cells, wild-type cells (Ly5a $^{+}$) responded more vigorously to infection than IFN γ R1 $^{-/-}$ cells (Ly5b $^{+}$), indicating that IFN γ directly stimulates CD8 $^{+}$ T cells, leading to their increased abundance (Fig. 3 B). The activation status of wild-type and IFN γ R $^{-/-}$ donor populations was similar, as indicated by increased expression of CD44 (Fig. 3 C), and both populations made IFN γ in re-

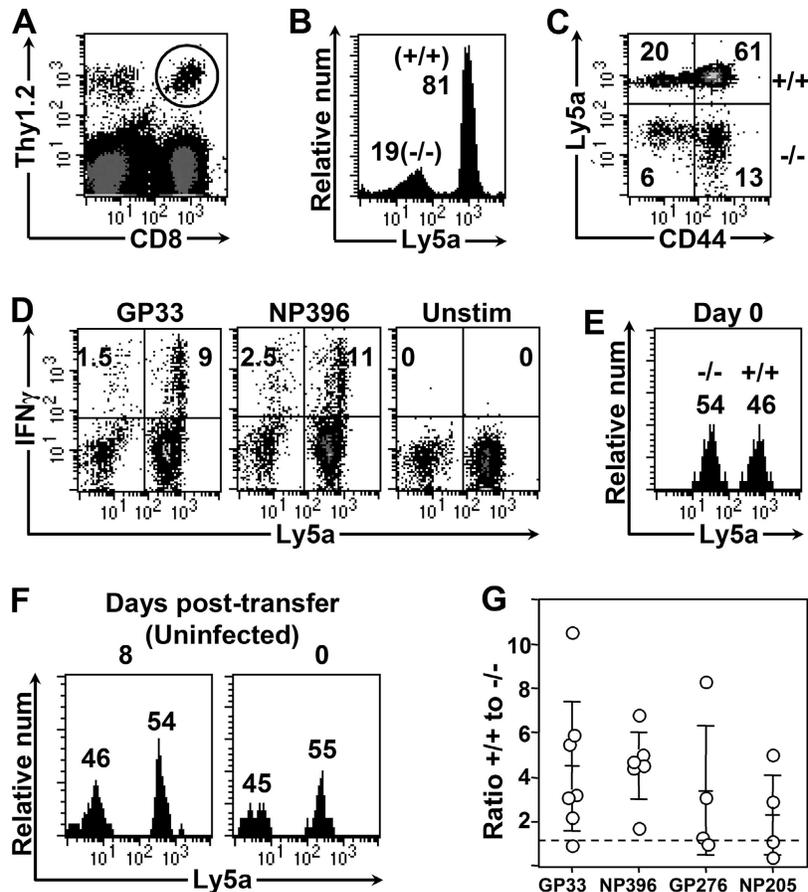


Figure 3. The stimulatory effect of IFN γ on CD8⁺ T cells requires that they express IFN γ R. Equivalent numbers of wild-type (Thy1.2⁺, Ly5a⁺) and IFN γ R^{-/-} (Thy1.2⁺, Ly5a⁻) CD8⁺ T cells were mixed and adoptively transferred intravenously into unirradiated, Thy1.1 mice. The recipient mice were infected and the responses of the donor (Thy1.2⁺) cells were measured 8 d after infection. (A) Representative dot plot shows the expanded donor cell populations (Thy1.2⁺), and these cells were gated, and are analyzed in B–G. (B) IFN γ R⁺ CD8⁺ T cells outnumber their IFN γ R⁻ counterparts by \sim 4:1. (C) CD44 expression on donor cells; the percentage of cells within each quadrant is shown. (D) The antigen-specific response of the donor cells was measured by ICCS. The numbers represent the

responding donor cells as percentage of all donor cells. (E) The proportion of wild-type and IFN γ R⁻ donor CD8⁺ T cells in these same mice at day 0 (measured in the blood before infection) is shown. (F) The percentage of donor cells that are Ly5a⁺ or Ly5a⁻ in an uninfected recipient mouse at day 0 (measured in blood) and 8 d later (measured in spleen). (G) The ratio of ^{+/+} to ^{-/-} donor cells is shown for four epitopes, based on ICCS. Each point represents an individual animal, and the means \pm SD are shown as horizontal lines. For each of four epitopes, the responses in ^{+/+} and ^{-/-} cell populations were compared using unpaired Student's *t* test. The *p*-values are: 0.0039 (GP₃₃); 0.0001 (NP₃₉₆); 0.021 (GP₂₇₆); and 0.038 (NP₂₀₅).

response to ex-vivo antigen stimulation (Fig. 3 D). The increased abundance of IFN γ R⁺ CD8⁺ T cells in response to infection was not due to differences in “take” immediately after adoptive transfer, because the relative proportion of cells transferred to the same mice was approximately equal immediately before infection (Fig. 3 E). Furthermore, these changes were due specifically to infection (and not preferential rejection of γ R^{-/-} cells) because mice given these cells but not infected showed no change in the proportion of wild-type to γ R^{-/-} donor cells at day 0 and 8 d later (Fig. 3 F). In this experimental model, both populations of donor cells are exposed to the same environment, so the differences in abundance are not due to differences in APC activation, IL-2 production, lymphoid architecture, or antigen load; we conclude that IFN γ acts directly on CD8⁺ T cells, thereby up-regulating

the primary CD8⁺ T cell response to infection. As noted above, previous analyses using IFN γ R^{-/-} mice indicated that the immunodominance hierarchy is influenced by IFN γ . To address whether IFN γ signaling affected the abundance of CD8⁺ T cells specific for a variety of epitopes, epitope-specific responses were measured in these mice (Fig. 3 G). As was seen for GP_{33–41} (presented by H-2Db), differences of two- to sixfold also were observed for CD8⁺ T cells specific to NP_{396–404} (H-2Db), GP_{276–286} (H-2Db), and NP_{205–212} (H-2Kb) indicating that both dominant and subdominant responses, and responses restricted to different MHC molecules, are increased when the T cells express IFN γ R1. To investigate whether IFN γ signaling in CD8⁺ T cells was autocrine in nature, a second dual adoptive transfer was performed, this time mixing Thy1.2, Ly5b IFN γ R^{-/-} cells (which express IFN γ R) and

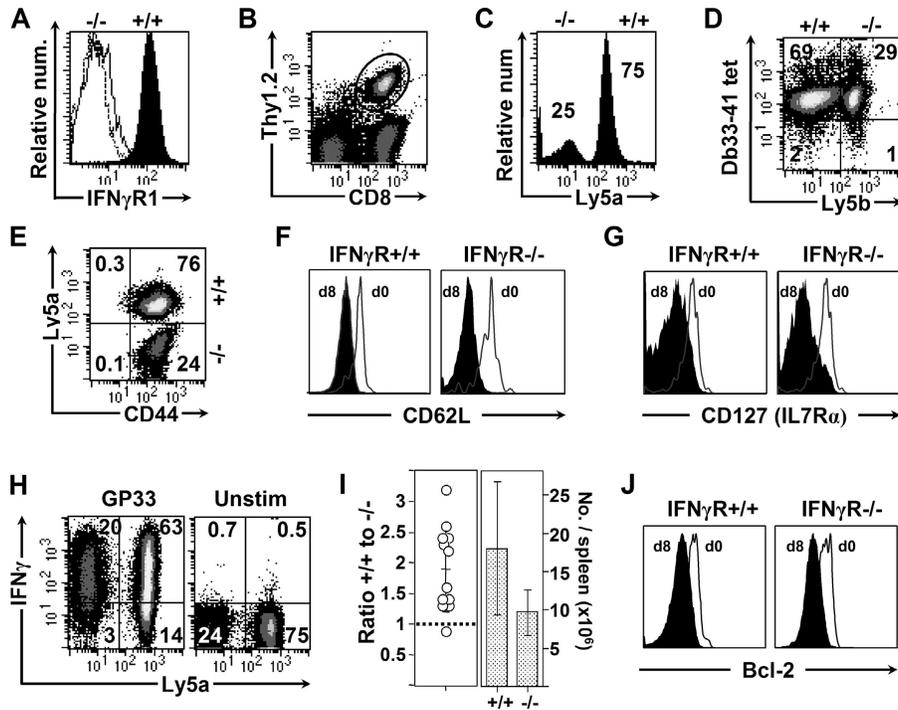


Figure 4. The stimulatory actions of IFN γ on CD8 $^{+}$ T cells occur during the course of virus infection. TcR-transgenic mice were bred as described in the text, to provide a source of IFN γ R1 $^{+}$ TcR-transgenic CD8 $^{+}$ T cells (Thy1.2 $^{+}$, Ly5a $^{+}$), and IFN γ R1 $^{-/-}$ TcR-transgenic CD8 $^{+}$ T cells (Thy1.2 $^{+}$, Ly5a $^{-}$). (A) Equal numbers of these IFN γ R1 $^{+}$ (shaded)- and IFN γ R1 $^{-/-}$ (unshaded)-transgenic CD8 $^{+}$ T cells were mixed and are shown (dotted line = isotype control). This 1:1 mix of IFN γ R1 $^{+}$ and IFN γ R1 $^{-}$ cells was injected into Thy1.1 recipients (each host mouse received a total of 6×10^4 TcR-transgenic CD8 $^{+}$ T cells), and the following day the recipient mice were infected. 8 d later, the responses of the two donor cell populations were analyzed by flow cytometry. (B) The expanded Thy1.2 TcR-transgenic donor cells are enclosed in an oval, and only these cells are analyzed in C–J. (C) There are threefold more IFN γ R $^{+}$ (Ly5a $^{+}$) cells than IFN γ R $^{-}$ cells. (D) The higher number of IFN γ R1 $^{+}$ cells is confirmed by Db33-41 tetramer

staining. (E–G) The activation status of the day 8 donor cells is similar, regardless of their expression of IFN γ R. The cells are CD44 hi , and at day 8, both donor populations show similar reductions in CD62L and CD127. (H) A similar proportion of each donor cell population produces IFN γ upon GP $_{33}$ peptide stimulation. (I) The ratio of $+/+$ to IFN γ R $^{-/-}$ -transgenic donor cells is summarized from 12 mice analyzed from three independent experiments (left); 11 of the 12 animals showed a ratio >1 (dotted line). The numbers of IFN γ R1 $^{+}$ and γ R $^{-/-}$ -transgenic cells were calculated from 12 mice (right). The averages \pm SD are indicated. The differences between the two groups was statistically significant, as indicated by a p -value of 0.0025, derived from a two-tailed paired Student's t test. (J) The antiapoptosis marker bcl-2 is reduced at day 8, to the same extent in the IFN γ R $^{+}$ and the IFN γ R $^{-}$ populations.

Thy1.2, Ly5a wild-type cells. IFN γ $^{-/-}$ CD8 $^{+}$ T cells expanded to the same extent as the cotransferred wild-type cells (unpublished data), indicating that autocrine production of IFN γ is not required for normal CD8 $^{+}$ T cell responses.

The stimulatory actions of IFN γ on CD8 $^{+}$ T cells occur during the course of virus infection

The reduced response of IFN γ R $^{-}$ T cells to LCMV infection, shown above, most likely reflects the lack of IFN γ signaling during infection. Nevertheless, other explanations are conceivable; e.g., although equivalent numbers of IFN γ R $^{-}$ and IFN γ R $^{+}$ CD8 $^{+}$ T cells were cotransferred into recipient mice, it is formally possible that the frequency of LCMV-specific precursors within each population might have been different. To ensure that equivalent numbers of LCMV-specific precursors were being transferred, we used TcR-transgenic mice. GP $_{33-41}$ TcR-transgenic mice were backcrossed either to wild-type mice expressing Ly5a (to provide a

source of TcR-transgenic IFN γ R $^{+}$, Thy1.2 $^{+}$, Ly5a $^{+}$ CD8 $^{+}$ T cells), or to the IFN γ R $^{-/-}$ mice (as a source of TcR-transgenic IFN γ R $^{-/-}$, Thy1.2 $^{+}$, Ly5a $^{+}$ cells), and 3×10^4 CD8 $^{+}$ T cells from each population were mixed (Fig. 4 A) and adoptively transferred into a normal Thy1.1 recipient mouse. By day 8 after infection, the Thy1.2-transgenic cells had expanded dramatically, to $1\text{--}4 \times 10^7$ cells per spleen, and were easily identifiable as a large proportion of the CD8 response (Fig. 4 B). Consistent with our findings using non-transgenic T cells, the IFN γ R $^{+}$ -transgenic T cells (Ly5a $^{+}$) responded better than the IFN γ R $^{-/-}$ -transgenic cells in the same host (Fig. 4 C). Tetramer staining reflected the numerical difference between IFN γ R $^{+/+}$ and IFN γ R $^{-/-}$ cells (Fig. 4 D). At 8 d after infection, all of the transferred cells, regardless of their IFN γ R status, showed a highly activated phenotype; they were CD44 hi , and showed down-regulation of CD62L and IL-7R α (Fig. 4, E–G) as well as increased expression of CD43 (1B11) and IL-2R β (CD122; unpublished

data). The similarities in phenotypic markers were paralleled by equivalent antigen responsiveness: 85% of IFN γ R⁻ cells, and 82% of IFN γ R⁺ cells, produced IFN γ in response to GP₃₃ peptide (Fig. 4 H). Thus, the presence or absence of the IFN γ R has little effect on CD8⁺ T cell activation or antigen-responsiveness, but it has a considerable effect on T cell abundance. The latter is summarized by results from 12 individual recipient mice (Fig. 4 I); IFN γ R⁺ cells almost always outnumbered their IFN γ R^{-/-} counterparts. The abundance of CD8⁺ T cells is determined by the balance between cell proliferation and cell death. The latter is thought to be mediated largely by apoptosis, and we considered the possibility that IFN γ might increase the levels of antiapoptotic proteins in CD8⁺ T cells. Therefore, we evaluated the expression of the antiapoptotic protein bcl-2, and found it to be similar in IFN γ R⁺ and IFN γ R⁻ CD8⁺ T cells (Fig. 4 J); the reduction observed at day 8 in wild-type cells is consistent with published data (18), and also occurs in IFN γ R⁻ cells.

In summary, we show here that IFN γ acts directly on CD8⁺ T cells, thereby increasing their abundance. Although the mechanism underlying the increase in T cell numbers remains to be determined, it is known that very early events can have a profound effect on the effector and memory phases of CD8⁺ T cell responses (19–21), and recent data indicate that IFN γ and its receptor play a key role in directing the very earliest stages of naive CD4⁺ cell commitment (22). Our data provide a possible explanation for the observations that (a) IFN γ modulates the immunodominance hierarchy of CD8⁺ T cell responses (7, 11), and (b) the rapid onset of IFN γ production by a CD8⁺ T cell may determine the ultimate abundance of its progeny (i.e., its immunodominance; 12). We propose that, early in the immune response, IFN γ produced by a CD8⁺ T cell can act directly upon the cell itself, or upon neighboring CD8⁺ T cells, enhancing their ultimate abundance. Consistent with this possibility, gene chip analyses of antigen-specific CD8⁺ T cells from the acute phase of the response show induction of a number of IFN γ -activated genes (23). The conclusion that IFN γ serves as a growth factor/costimulatory molecule for T cell responses is at odds with previous studies showing that IFN γ suppresses T cell responses. Increased T cell responses occur in IFN γ ^{-/-} mice given rLM(ActA⁻) or *Mycobacterium* (7, 8), but this could reflect delayed pathogen clearance, resulting in a greater antigen load that stimulates continued T cell proliferation. Others have shown that CD8⁺ T cells in LCMV-infected IFN γ R⁻ mice are hyperproliferative; although in that model, there is a delay in virus clearance that may modify the T cell response (24). Can our findings be integrated with current dogma? The biological effects of several cytokines differ depending on the time at which they are expressed. For example, IL-2 can either enhance T cell responses or inhibit them, depending on when it is administered during virus infection (25, 26), and IFN α / β can enhance T cell responses (27) but also can induce T cell apoptosis (28). Perhaps the effect of IFN γ on CD8⁺ T cells also changes

over the course of an ongoing immune response, as suggested by studies of transformed human T cells (29); its early effects may be stimulatory (as shown here), and its later effects suppressive. Our demonstration that IFN γ may stimulate, rather than abrogate, T cell responses may provide an explanation for the deleterious effects of this cytokine when used to treat multiple sclerosis (30), and counsels caution for its future use in autoimmune diseases, and in other circumstances in which immunostimulation might be harmful.

MATERIALS AND METHODS

Mice and virus. C57BL/6 mice were purchased from The Scripps Research Institute (TSRI) breeding facility. IFN γ ^{-/-} mice and IFN γ R1^{-/-} mice (both strains backcrossed 10 generations to C57BL/6), and C57BL/6 mice congenic for Thy1.1 were purchased from Jackson ImmunoResearch Laboratory. C57BL/6.Ly5a mice were provided by Dr. Charlie Surh (TSRI). P14 TCR-transgenic mice specific for the LCMV epitope GP₃₃₋₄₁ were crossed to C57BL/6.Ly5a mice to generate TcR-transgenic IFN γ R⁺ Ly5a mice; and to C57BL/6 IFN γ R1^{-/-} mice to generate TcR-transgenic IFN γ R⁻ Ly5b mice. Mice were infected by intraperitoneal administration of 2 × 10⁵ plaque forming units of LCMV, Armstrong strain. All experiments were approved by the TSRI Animal Care and Use Committee.

Flow cytometry. Spleen cells were stained directly ex vivo with anti-CD8 (clone 53–6.7), anti-Thy1.2 (CD90.2 clone 53–2.1), anti-CD44 (clone IM7), Ly6A/E (clone D7), and anti-Ly5.1 (Ly5a, clone A20; eBioscience). Antibodies used for staining IFN γ R1 (rat, clone GR20) and the corresponding isotype control antibodies were purchased from BD-PharMingen. The intracellular staining (ICCS) assay was performed as described previously (11). In vivo cytotoxicity was assayed by labeling naive B6.Ly5a splenocytes with either 3 or 0.3 μM CFSE (5,6-carboxy-fluorescein diacetate succinimidyl ester; Molecular Probes). The CFSE^{hi} cells were coated with the GP₃₃ peptide, and the CFSE^{lo} cells were left uncoated. After extensive washing, equal numbers of the CFSE^{hi} and CFSE^{lo} cells were mixed, and transferred into either naive recipients or day 8-infected mice. After 8 h, Ly5a⁺ cells were identified by flow cytometry; the percent killing was calculated as 100 – {[(% peptide coated in infected / % uncoated in infected) / (% peptide coated in uninfected / % uncoated in uninfected)] × 100}. Cell staining was analyzed by four-color flow cytometry at the TSRI core facility using a BD Biosciences FACSCALIBUR and Cell Quest software.

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